Biotransformation of acetamiprid by the white-rot fungus Phanerochaete sordida YK-624

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Research Paper

Biotransformation of acetamiprid by the white-rot fungus *Phanerochaete sordida* YK-624

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Abstract

Acetamiprid (ACE) belongs to the neonicotinoid class of systemic broad-spectrum insecticides, which are the most highly effective and largest selling insecticides worldwide for crop protection. As neonicotinoid insecticides persist in crops, biotransformation of these insecticides represents a promising approach for improving the safety of foods. Here, the elimination of ACE from liquid medium by the white-rot fungus *Phanerochaete sordida* YK-624 was examined. Under ligninolytic and non-ligninolytic conditions, 45% and 30% of ACE were eliminated, respectively, after 15 days of incubation. HR-ESI-MS and NMR analyses of a metabolite identified in the culture supernatant suggested that ACE was *N*-demethylated to (*E*)-N^1-[(6-chloro-3-pyridyl)-methyl]-N^2-cyano-acetamidine, which has a much lower toxicity than ACE. In addition, we investigated the effect of the cytochrome P450 inhibitor piperonyl butoxide (PB) on the elimination of ACE. The elimination rate of ACE by *P. sordida* YK-624 was markedly reduced by the addition of either 0.01 and 0.1 mM PB to culture medium. These results suggest that cytochrome P450 plays an important role in the *N*-demethylation of ACE by *P. sordida* YK-624.

Keywords: Acetamiprid · Detoxification · *Phanerochaete sordida* YK-624 · *N*-demethylation · White-rot fungi

Introduction

Neonicotinoid insecticides, which are one of the most important classes of commercial insecticides worldwide, are systemic in plants and animals and are used to manage crop pests and control fleas on cats and dogs (Arther et al. 1997; Jacobs et al. 1997; Yamamoto and Casida 1999). Acetamiprid (ACE; Fig. 1) belongs to the class of chloronicotinyl neonicotinoid insecticides and is used to control Hemiptera, particularly aphids, Thysanoptera, and Lepidoptera, on a wide range of crop species (Mateu-Sanchez et al. 2003; Tokieda et al. 1997). In recent years, ACE residues in crops are receiving considerable attention due to their potential toxicity to humans (Pramanik et al. 2006; Sanyal et al. 2008), and methods for the biotransformation of neonicotinoids are being actively researched. A number of microorganisms that are capable of degrading neonicotinoids have been identified, including the yeast *Rhodotorula mucilaginosa* strain IM-2, which is able to hydrolyze ACE and thiacloprid, and the bacterium *Stenotrophomonas maltophilia* CGMCC 1.1788, which is able to hydroxylate imidacloprid and *N*-demethylate ACE (Chen et al. 2008; Dai et al. 2006; Dai et al. 2010).

Lignin-degrading white-rot fungi, which have the unique ability to degrade lignin

35 to the level of CO_2 (Kirk and Farrell 1987), and their ligninolytic enzymes have also
36 attracted interest for the biotransformation of contaminants because of their industrial
37 potential for degrading recalcitrant environmental pollutants, such as polychlorinated
38 dibenzodioxin (Kamei et al. 2005), lindane (Bumpus et al. 1985), chlorophenols (Joshi
39 and Gold 1993), and polycyclic aromatic hydrocarbons (Bezalel et al. 1996; Collins et
40 al. 1996). More recently, we reported that the white-rot fungus *Phanerochaete sordida*
41 YK-624 produces manganese peroxidase (MnP) that is capable of detoxifying aflatoxin
42 B_1, which is a frequent contaminant of food products (Wang et al. 2011).
43 Here, we examined the elimination of ACE by *P. sordida* YK-624 in order to
44 develop a bioremediation system for ACE-contaminated foods. We also detected the
45 metabolite from ACE and proposed a metabolic pathway for the metabolism of ACE by
46 *P. sordida* YK-624. This represents the first report describing the biotransformation of
47 ACE by a white-rot fungus.

Materials and methods

48 Fungal strain and culture conditions
49 *P. sordida* YK-624 (ATCC 90872), which has been isolated from rotted wood

50 (Hirai et al. 1994), was used in the present study. The fungus was maintained on potato
51 dextrose agar (PDA) slants at 4°C.

52 Chemicals

53 ACE and piperonyl butoxide (PB) were purchased from Wako Pure Chemical
54 Industries (Osaka, Japan). All other chemicals were analytical-pure grade and were used
55 without further purification.

56 Fungal treatment of ACE

57 Nitrogen-limited (NL) medium described by Tien and Kirk (1988) and potato
58 dextrose broth (PDB) medium (4% potato starch, 2% dextrose, pH 4.5) were used for
59 ACE elimination experiments. The fungus was incubated on a PDA plate at 30 °C for 3
60 days, and then 10-mm-diameter disks were punched out from the growing edge of
61 mycelium. Two disks were each placed into a 100-mL Erlenmeyer flask containing 10
62 mL of liquid medium (NL or PDB medium, pH 4.5). After statically incubating the
63 flasks at 30 °C for 7 days, 100 μL of 1 mM ACE (final concentration: 10 μM) was
64 added to the cultures, which were then further incubated for 5, 10, 15 and 20 days (each
65 in triplicate). The culture was filtrated with a 0.2-μm membrane filter, and the filtrate

66 was then subjected to high-performance liquid chromatography (HPLC) for the
67 quantification of ACE under the following conditions: column, Wakosil-II 5C18HG (4.6
68 x 150 mm; Wako Pure Chemical Industries); mobile phase, 30% MeOH aq.; flow rate,
69 0.5 mL/min; and UV wavelength, 246 nm.

70 Metabolite identification

71 Inoculated cultures (5 L NL medium) of *P. sordida* YK-624 were prepared under
72 the conditions described above. After static incubation at 30 °C for 7 days, 5 ml of 100
73 mM ACE (final concentration: 100 μM) was added to these cultures. The cultures were
74 further incubated for 15 days and then filtrated with a 0.2-μm membrane filter. Distilled
75 water (100 mL) was added to the filtrate after evaporation to dryness, and the resulting
76 solution was extracted twice with 100 mL ethyl acetate (EtOAc). The EtOAc extract
77 was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue
78 was cleaned on a silica gel flash column chromatography (silica gel 60N, φ40 × 600
79 mm) and eluted with dichloromethane/EtOAc/MeOH (10/0/0, 9/1/0, 8/2/0, 7/3/0, 5/5/0,
80 3/7/0, 0/10/0, 0/9/1, 0/8/2, 0/7/3, 0/5/5, and 0/0/10; vol/vol/vol) to obtain 14 fractions.
81 Each fraction was analyzed by thin-layer chromatography (TLC), high performance
82 liquid chromatography (HPLC), high-resolution-electrospray ionization-mass

(HR-ESI-MS), and ^{1}H- nuclear magnetic resonance (NMR). Silica gel plates (Merck F254; Merck, Darmstadt, Germany) and silica gel 60N (Merck 100-200 mesh; Merck) were used for analytical TLC and flash column chromatography. The metabolite was further separated by HPLC (column: Develosil C30-UG-5; Nomura Chemistry, Seto, Japan) using 70% MeOH. The purified metabolite was analyzed by HR-ESI-MS and NMR, including correlation spectroscopy (COSY), hetero-nuclear multiple quantum coherence (HMQC), and hetero-nuclear multiple-bond connectivity (HMBC) spectroscopies. The HR-ESI-MS data were measured using a JMS-T100LC mass spectrometer. ^{1}H-NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz.

Cytochrome P450 inhibitor experiment

After pre-culturing *P. sordida* YK-624 for 5 days, ACE (final concentration: 10 μM) and the cytochrome P450 inhibitor PB (final concentration: 0, 0.01, and 0.1 mM) were added to cultures. The cultures were further incubated for 5, 10, and 15 days, and each culture was then subjected to HPLC for the quantification of ACE, as described above **(**column, Wakosil-II 5C18HG; mobile phase, 30% MeOH aq.; flow rate, 0.5 mL/min; and UV wavelength, 246 nm.**)**. All experiments were performed in triplicate.

Results

Elimination of ACE by *P. sordida* YK-624

In the present study, NL and PDB media were used for the elimination experiment of ACE. When *P. sordida* YK-624 was cultured in NL medium, ACE was reduced by approximately 45% after 20 days of incubation (Fig. 2). In PDB medium, the eliminated concentration of ACE was approximately 28% after 20 days of incubation. Moreover, purified lignin peroxidase (LiP) and MnP from *P. sordida* YK-624 could not degrade ACE (data not shown). These results suggested that *P. sordida* YK-624 had a higher ability for the elimination of ACE in NL medium and that ligninolytic enzymes (LiP and MnP) were not involved in the elimination of ACE, as ACE was also eliminated under the non-ligninolytic conditions provided by PDB medium. In fact,

Identification of the metabolite from ACE

As shown in Fig. 3, main metabolite was detected in HPLC analysis of 10-day NL culture fluid inoculated with *P. sordida* YK-624, and minor metabolites were hardly detected. To determine the structure of the metabolite produced during the degradation

114 of ACE, 15-day cultures of *P. sordida* YK-624 in NL medium supplemented with 100
115 μM ACE were subjected to TLC and HPLC. The purified metabolite was then subjected
116 to HR-ESI-MS analysis, which yielded a molecular ion at *m/z* 231.0428 $[M+Na]^+$
117 (calculated for $C_9H_9ClN_4Na$, 231.0413), indicating that the molecular formula of this
118 compound was $C_9H_9ClN_4$. This formula suggested that the metabolite might be a
119 demethylated form of ACE. The structure of the purified metabolite was further
120 characterized by NMR analyses. Table 1 lists the chemical-shift assignment data for the
121 metabolite of ACE. HMBC correlations (Fig. 4) (H-7/C-2, H-7/C-3, H-7/C-4; H-7/C-9,
122 H-13/C-9) confirmed that the metabolite was *N*-demethylated ACE,
123 (*E*)-N^1-[(6-chloro-3-pyridyl)-methyl]-N^2-cyano-acetamidine, which is commonly known
124 as IM 2-1 (Fig. 1).

125 Effect of cytochrome P450 inhibitors

126 The effect of cytochrome P450 inhibitors on the elimination of ACE by *P. sordida*
127 YK-624 were investigated using PB. The elimination rate of ACE after the addition of
128 0.01 and 0.1 mM PB into cultures of *P. sordida* YK-624 is shown in Fig. 5. In contrast
129 to the elimination rate of ACE in cultures without PB, markedly lower elimination
130 activity of ACE was observed in cultures containing PB. In the presence of 0.01 and 0.1

131 mM PB, only 16% and 7% of ACE were eliminated after 15 days of incubation,
132 respectively, compared to 45% in cultures lacking PB.

Discussion

133 White-rot fungi are capable of degrading a wide variety of recalcitrant aromatic
134 compounds, including polymeric lignin and environmentally persistent pollutants.
135 However, prior to the present study, the degradation of neonicotinoid insecticides by
136 white-rot fungi had not been reported. Here, we demonstrated the elimination of ACE
137 from liquid cultures of the white-rot fungus *P. sordida* YK-624. Under ligninolytic and
138 non-ligninolytic conditions, 45% and 30% of ACE, respectively, were eliminated after
139 15 days of incubation (Fig. 2). Although the fungal growth in PD media is much faster
140 than in NL media, the elimination rate of ACE in PD media was almost same or lower
141 than in NL medium. Moreover, no decrease was observed in PD medium containing 10
142 μM ACE without fungal inoculation for 20 days. These results suggest that ACE was
143 biotransfored to other compounds by fungal treatment, not abiotic elimination or
144 adsorption on biomass.
145 We detected the generation of the ACE metabolite IM 2-1 by *P. sordida* YK-624

146 (Fig. 3). The metabolic pathway of ACE has been studied in honeybee, mice, spinach,
147 and soil bacteria (Brunet et al. 2005; Ford and Casida 2006; Ford and Casida 2008;
148 Tokieda et al. 1999). In these studies, the main metabolic pathway involved the
149 *N*-demethylation of ACE to yield IM 2-1. In honeybee, the nitromethylene bond of IM
150 2-1 might be subsequently oxidized to yield 6-chloronicotinic acid (Brunet et al. 2005).
151 In addition to the metabolite IM 2-1, we also detected other compounds by HPLC and
152 TLC analyses of *P. sordida* YK-624 culture supernatant. However, we were unable to
153 determine the structures of these additional metabolites due to their low concentrations.
154 The lipophilic *N*-methyl group of neonicotinoids plays an important role in the
155 bioefficacy of these insecticides, with the loss of this group reducing the insecticidal
156 activity by more than 10-fold or even leading to complete inactivation (Chen et al.
157 2008). Although ACE is reported to have a relatively high toxicity in the honeybee, with
158 an LD_{50} value of 7.1 μg/bee, the metabolite IM 2-1 resulted in no mortality at 50 μg/bee
159 (Iwasa et al. 2004). Since the metabolite IM 2-1 has lower toxicity than ACE, the
160 detoxification of ACE is possible using *P. sordida* YK-624.
161 The functional diversity of cytochrome P450s in white-rot fungi has been studied
162 because cytochrome P450s play an important role on degrading a wide variety of
163 recalcitrant aromatic compounds (Hiratsuka et al. 2001; Ichinose et al. 1999; Masaphy

et al. 1996). Recently, Hata et al. (2010) suggested that hydroxylation catalyzed by cytochrome P450 in *P. sordida* YK-624 might be involved in the elimination and detoxification of diclofenac and mefenamic acid. The involvement of cytochrome P450 in the *N*-demethylation of a few drugs has also been reported in human cells (Ghahramani et al. 1997; Sutton et al. 1997). Our present study showed that the elimination of ACE was efficiently inhibited by the addition of PB, which is a common inhibitor of cytochrome P450 and is often used for demonstrating whether a reaction is catalyzed by cytochrome P450 enzymes (Kamei et al. 2005; Mori and Kondo 2002; Mori et al. 2003). Since *N*-demethylation of ACE was inhibited by PB, we propose that cytochrome P450s are involved in the *N*-demethylation of ACE.

In conclusion, we have described for the first time the biotransformation of ACE by a white-rot fungus.

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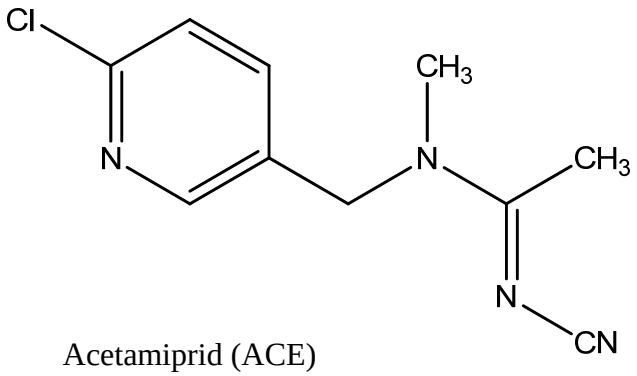
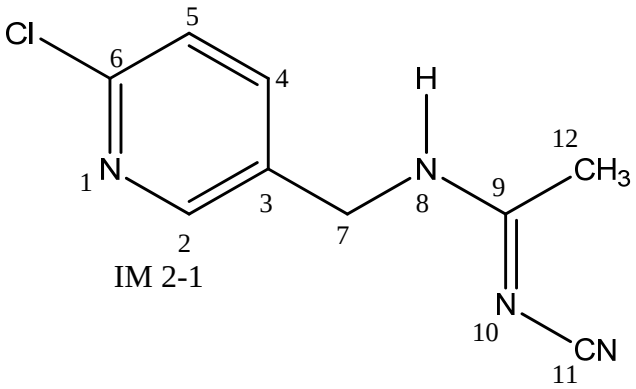


Acetamiprid (ACE)



IM 2-1

Fig. 1 Structures of acetamiprid and the metabolite IM 2-1

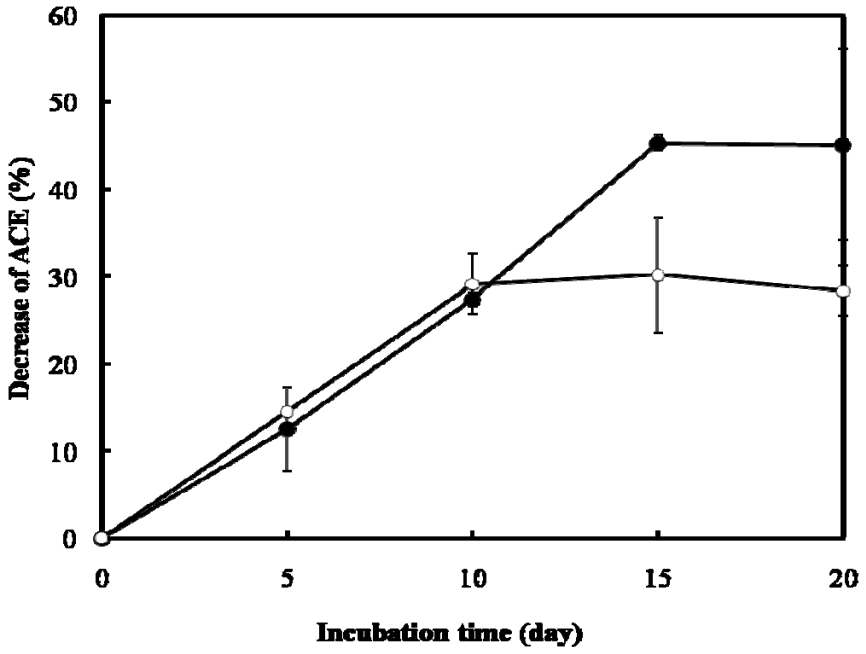


Fig. 2 Time course for ACE elimination by *P. sordida* YK-624. Closed circles, NL medium; open circles, PDB medium. Values are the means ± SD of triplicate samples

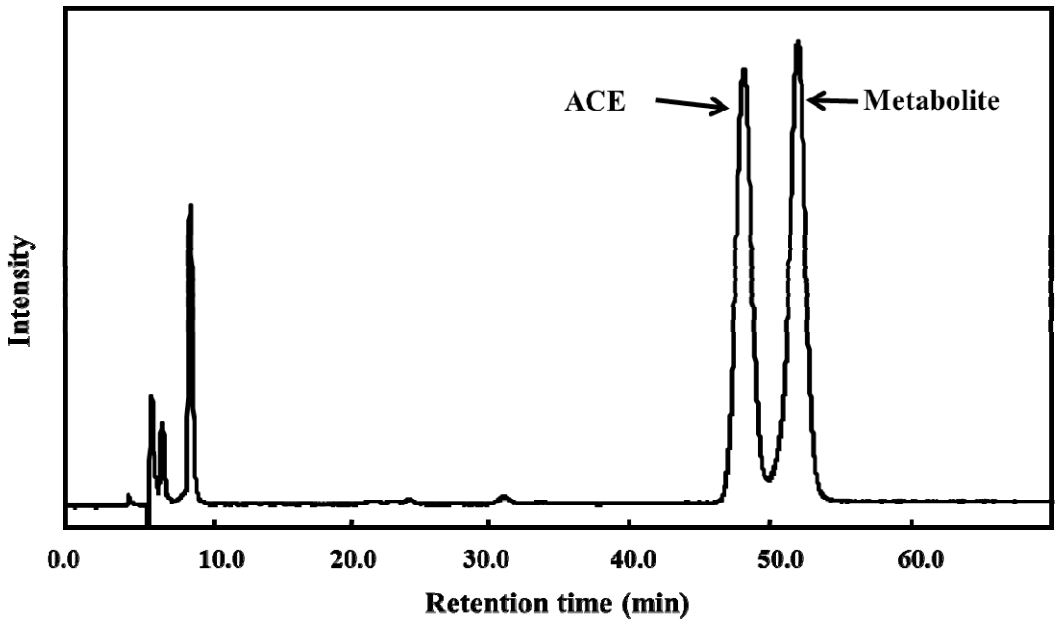


Fig. 3 Detection of the ACE metabolite in the 10-day NL culture fluid by HPLC. These compounds were detected by HPLC under the following conditions: column, Wakosil-II 5C18HG; mobile phase, 30% aqueous MeOH; flow rate, 0.5 mL/min; and detection wavelength, 246 nm.

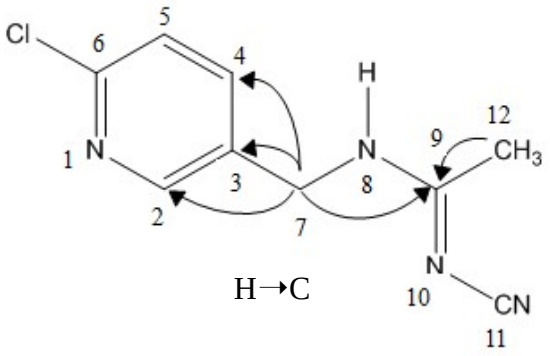


Fig. 4 HMBC correlations of the identified ACE metabolite

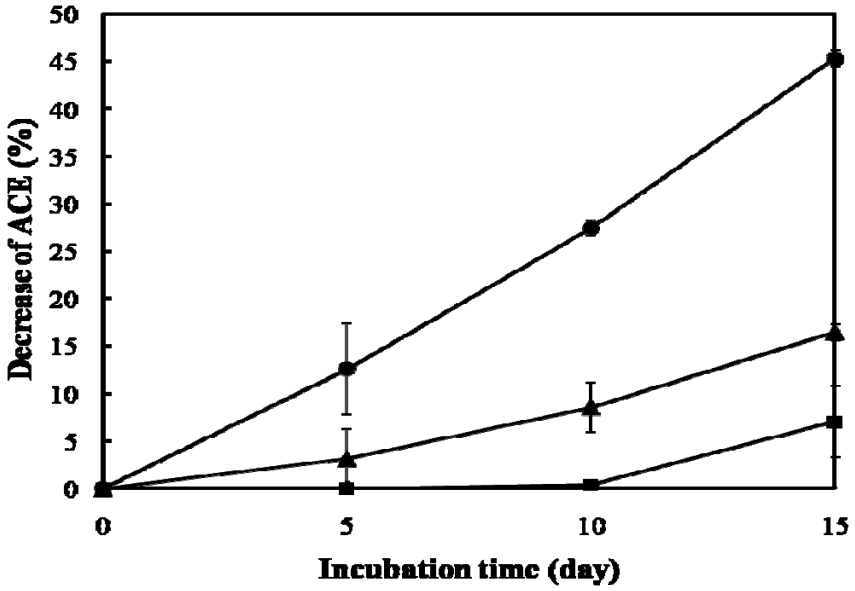


Fig. 5 Effect of the cytochrome P450 inhibitor PB on the elimination of ACE by *P. sordida* YK-624 in NL medium. Circles, without PB; triangles, 0.01 mM PB; squares, 0.1 mM PB. Values are the means ± SD of triplicate samples

Table 1 NMR data for the metabolite of ACE in CD_3OD

Position	^{1}H	^{13}C
	δ_H (mult, *J* in Hz)	δ_C
2	8.22 (d 2.6)	149.9
3	-	124.0
4	7.69 (dd 8.2, 2.6)	139.2
5	7.34 (d 8.2)	132.4
6	-	148.7
7	4.39	41.6
9	-	172.5
11	-	117.5
12	2.21	19.1